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Apoptosis Is Inversely Related to Necrosis and Determines Net Growth in Tumors Bearing Constitutively Expressed *myc*, *ras*, and HPV Oncogenes

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Immortalized rat fibroblasts were transfected with expression plasmids containing a mutated human Ha-ras (T24) oncogene, human c-myc, HPV 16 or 18 genomes, or combinations of these. Cell proliferation rates in vitro of the resulting 13 transformed lines were closely similar but apoptotic rates in vitro varied over a 60-fold range and correlated inversely with rates of population expansion in culture. To determine whether such differences in susceptibility to apoptosis affected the pattern of tumor growth in vivo, the transfected lines were injected subcutaneously into immunesuppressed mice producing fibrosarcomas in which prevalence of apoptosis and mitosis, extent of necrosis, and net growth rate were measured. Cell lines with high apoptotic rates in vitro tended to generate slowly growing tumors with high ratios of apoptosis to mitosis and little necrosis. The three most extreme examples of this phenotype all resulted from single transfections with c-myc. Lines with low apoptotic rates in vitro generated rapidly expanding tumors with high mitotic rates, extensive necrosis, and little apoptosis relative to mitosis, even in the compromised zone at the edge of necrotic regions. The four fastest-growing tumors all contained a T24-ras oncogene. The results suggest that oncogene expression determines intrinsic apoptotic rates and in this way may significantly influence the net growth rate and extent of necrosis in tumors. (Am J Pathol 1994, 144:1045–1057)

Many factors influence the rate of tumor growth. Tumor cell proliferation has been extensively studied but does not correlate well with overall growth rate.[1] Part of the reason for this is that tumor growth results from the balance of cell gain with cell loss, and quantitative data indicate that cell loss from growing tumors is considerable. Overall cell loss can be measured as the cell loss factor (CLF), which expresses the difference between the growth expected of a tumor on the basis of its cell proliferation rate and the observed growth rate.[2] CLFs of 1 indicate that cell loss balances cell gain exactly and CLFs of 0 indicate that all proliferating cells are retained alive. Recorded values of CLFs in rodent sarcomas and carcinomas are in the range of 0.65 to 0.78, whereas for human bronchial, colorectal carcinomas, and malignant melanomas they are higher still at 0.73 to 0.96.[2–5]

The two main mechanisms of cell loss from growing tumors are apoptosis and necrosis.[6,7] Apoptosis is genetically programmed and usually affects single cells surrounded by viable neighbors. In contrast, necrosis results from severe nonphysiological perturbation of the cellular environment and often occurs in confluent zones within tumors. The topography of necrosis in tumors is a complex function, a major determinant of which is the rate of proliferation of tumor cells relative to the process of angiogenesis, itself induced by tumor-derived growth and angiogenic factors.[2–4,8] Blood flow within tumors is notably heterogeneous and is not always directly proportional to the anatomic extent of their vasculature.[9] Local regions of rapid tumor expansion may produce zones of hypoxia resulting in necrosis. This has been observed at a strikingly constant distance from blood vessels or oxygen supply as within experimental tumor "spheroids" *in vitro* and "corded carcinomas" *in*

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vivo.[3,4,8–13] However, despite the histologically conspicuous appearance of necrosis, it is by no means established that it can account for the high tumor CLFs observed. Apoptosis is less conspicuous histologically because it is swiftly completed, leaves no residua, and occurs in a scattered topographical distribution. It is readily calculated that a surprisingly small proportion of apoptotic cells visualized in a tissue section can represent very substantial cell loss[14,15] but there are few quantitative reports on apoptosis in tumors.[4,7,16–21]

The patterns of cell death within the microenvironment of growing tumors are important, because they express fundamental features of tumor biology and therefore may relate to prognosis. Diagnostic histopathologists are familiar with the somewhat counterintuitive notion that tumors with a large proportion of necrosis have a bad prognosis.[22] The tumor microenvironment may also influence the response to therapy, because hypoxia diminishes the cytotoxicity of ionizing radiation[23,24] and alters the expression and efficiency of drug metabolizing enzyme systems.[3,25] Furthermore, local permeation of systemic chemotherapeutic agents must be affected by the detailed topographical relationships between tumor cells and the microcirculation.[26] Moreover, many recent observations of cells *in vitro* show that susceptibility to apoptosis is regulated by the expression of oncogenes and oncosuppressor genes and that this may have much to do with their sensitivity to cytotoxic agents of various types.[27–31]

In this paper we explore the possibility that apoptosis may be important in determining net tumor growth and patterns of tumor necrosis by comparing parameters of growth *in vivo* with those *in vitro*. We describe differences in the apoptotic rates of a family of cell lines derived from a single parental rat fibroblast line by transfection with *myc*, *ras* and HPV genes. We show that these differences in apoptosis are responsible for the widely divergent overall growth rates of the lines *in vitro* and are conserved *in vivo* where they contribute to differences in tumor growth rate. Differences in susceptibility to apoptosis correlate inversely with the tendency to develop necrosis. The data provide evidence that oncogenes regulate apoptosis in tumors and by this means determine important elements of the growth patterns of these tumors *in vivo*.

Materials and Methods

Construction of the Cell Lines

The immortal but untransformed Fischer rat lung fibroblast line 208F[32] was transfected with expression vectors containing human c-*myc*, mutationally activated human c-Ha-*ras*l (T24-*ras*), and HPV 16 and 18 genomes as described elsewhere.[33–35] In all, 14 cell lines were investigated (Table 1): the control untransfected parent (208F), 3 sublines transfected with c-*myc* (M1, M7, and M8), 3 transfected with T24-*ras* (T1, T2, and T3), 3 c-*myc*/T24-*ras* co-transfectants (MT7, MT9, and MT10), and 4 HPV-containing transfectants of which 1 received HPV 16 alone (H16), 1 HPV 18 alone (H18), and 2 were HPV/T24-*ras* co-transfectants (H16R, H18R). The MT7 cell line was derived from T1 cells by electroporation with the c-*myc* expression plasmid pHRMCGM1.[35] MT9 and MT10 were co-transfectants both derived from 208F by simultaneous electroporation with c-*myc* and T24-*ras* expression plasmids (pHRHMCGM1 and pHRHO5T1).[35] H16 and H18 were derived by electroporation of 208F with pJ4Ω16/pSV2Neo and pJ4Ω18/pSV2Neo, respectively,[34] whereas H16R and H18R were derived by co-transfection with pJ4Ω16/pHO5T1 and pJ4Ω18/pHO5T1, respectively.[33,34] These mixed transfectants were selected with either hygromycin B or G418 (geneticin) and single colonies isolated as clones. The presence of transfected DNA was confirmed by Southern hybridization analysis or polymerase chain reaction using specific primers (data not shown).[35–38] The c-*myc* RNA expression was confirmed by reverse transcription-polymerase chain reaction using exon connection primers and also by RNA dot blot hybridization analysis (data not shown) and enhanced $p21^{ras}$ expression was confirmed by immunocytochemistry.[35]

Cell Growth In Vivo

Groups of 6 to 11 immunosuppressed CBA mice received 10 million cells by subcutaneous injection[39] for each cell line. After 12 days animals were killed and autopsied. At this stage most animals appeared healthy but those injected with T1 and MT7 cells bore large tumors and pilot experiments showed that if left undisturbed they died a few days later.[39] In a separate series of experiments mice injected with M1 cells were kept for periods of up to 70 days to generate tumors of comparable size to the fast-growing T1 tumors at 12 days. At autopsy, three mutually perpendicular tumor diameters were measured and multiplied together to express tumor size as a "box volume" (cm^3). Tumors were bisected and one-half analyzed histologically after fixation in periodate-lysine-paraformaldehyde-dichromate, preparation of paraffin sections, and staining with hematoxylin and eosin. The extent of tumor necrosis was assessed

semiquantitatively on a scale of 0 to 4, where 4 represented >75% necrosis (by area), 3 represented 50 to 75% necrosis, 2 represented 25 to 50% necrosis, 1 represented small scattered islands of necrosis <25% total area, and 0 stood for absence of necrosis. Six tumors from each cell line were assessed for extent of necrosis and the mean necrosis score was calculated and multiplied by 10. The number of mitotic and apoptotic figures were identified by standard criteria[14,15,40] and counted in the same fields over 10 high power fields selected within viable portions of each tumor. The raw data for apoptotic counts and mitotic counts were combined to form ratios of apoptosis/mitosis (A/M). This corrects for differences in density of apoptosis and mitosis per field that derive solely from factors such as cell size and the density of cell packing within the tumors. A/M ratios and rates of apoptosis in culture were log transformed to render the data near-Gaussian in distribution to allow statistical comparison by the Student's *t*-test. In tumors this was based on at least 60 data points (10 high power fields from each of at least 6 tumors from each cell line).

Growth In Vitro

This was studied in cultures in 75-cm^2 flasks seeded with 8×10^5 cells. Apoptosis was measured by collecting media at 48 hours. Cell bodies released from the monolayer into the media (released cell bodies, RCB) were resuspended in a known volume of phosphate-buffered saline and the total numbers of RCB were counted using an improved Neubauer hemocytometer. The 20 µl of this sample was mixed with an equal volume of 10 µg/ml acridine orange on a glass slide and viewed under ultraviolet light. The 100 to 200 cellular bodies were counted and identified as viable, apoptotic, or necrotic, differentiated by their characteristic morphological appearances.[14,15] This was used to calculate the proportion of apoptotic bodies (%A) and viable (%V) cells comprising the RCB. The monolayer was carefully harvested by trypsin-EDTA digestion[35] and counted to give the total number of monolayer cells (MC), including any residual cells collected by a thorough phosphate-buffered saline wash of the flask. The apoptotic index (AI) was calculated as a measure of the production of apoptotic bodies per 100 attached cells over 48 hours using the following equation: $AI = [\%A \times RCB \times 100]/[(\%V \times RCB) + MC]$ and a mean AI for each cell line was derived from 5 to 10 replicate experiments.

Cell Cycle Analysis

This was performed on trypsinized, washed cell suspensions using a standard protocol for flow cytometric measurement of the nuclear DNA content.[4] Cells were analyzed while growing in maximal growth phase in culture. The computer software program, SFITS (Coulter Electronics), was used to determine the proportion of cells in each phase of the cell cycle. The growth fraction was calculated as the proportion of cells in S plus G2/M phases of the cell cycle (9 to 10 experiments per cell line). The ratio of the mean monolayer cell numbers at 48 and 24 hours (termed population expansion, PE) was used as a single measure of the proportional increase in cell number over 1 day. This allowed comparison of the increases in net cell numbers between different cell lines while in maximal growth phase (3 to 9 experiments per cell line).

Results

Growth In Vitro

Morphological Phenotype

The parental 208F cell line demonstrated topoinhibition of growth in culture by forming a flat confluent monolayer of nonrefractile, nontransformed polygonal cells. Controls for subsequent experiments included untransfected 208F cells, cells electroporated with no DNA, or transfected with either the drug selection vector only (pSV2Neo) or viral enhancer/promotor sequences and drug selection vector only (pHomer5), all of which showed no evidence of morphological transformation. The three c-*myc* transfected cell lines all demonstrated morphological features of a mild degree of transformation. The flat, spindle-shaped cells were more refractile than the parental line and there was tight side-to-side clustering of cells producing a fascicular appearance with formation of small foci (2 to 3 cells high). The c-*myc* transcripts were previously estimated at approximately 0.5% of total cellular mRNA in M1 cells, approximately 18-fold higher than untransfected 208F controls,[39] and semiquantitative dot blot analysis of *myc* RNA levels in M7 and M8 showed approximate equivalence to those in M1 cells (unpublished data). The T24-*ras*-containing lines T1, T2, T3, MT7, H16R, and H18R all showed a markedly transformed phenotype. Their cells were tubular or spindle shaped, strongly refractile, and piled up to more than three cells' depth in large foci (Figure 1). T24-*ras* transcripts were previously estimated at around 0.8% of total cellular mRNA in T1 cells, approximately 20-fold greater

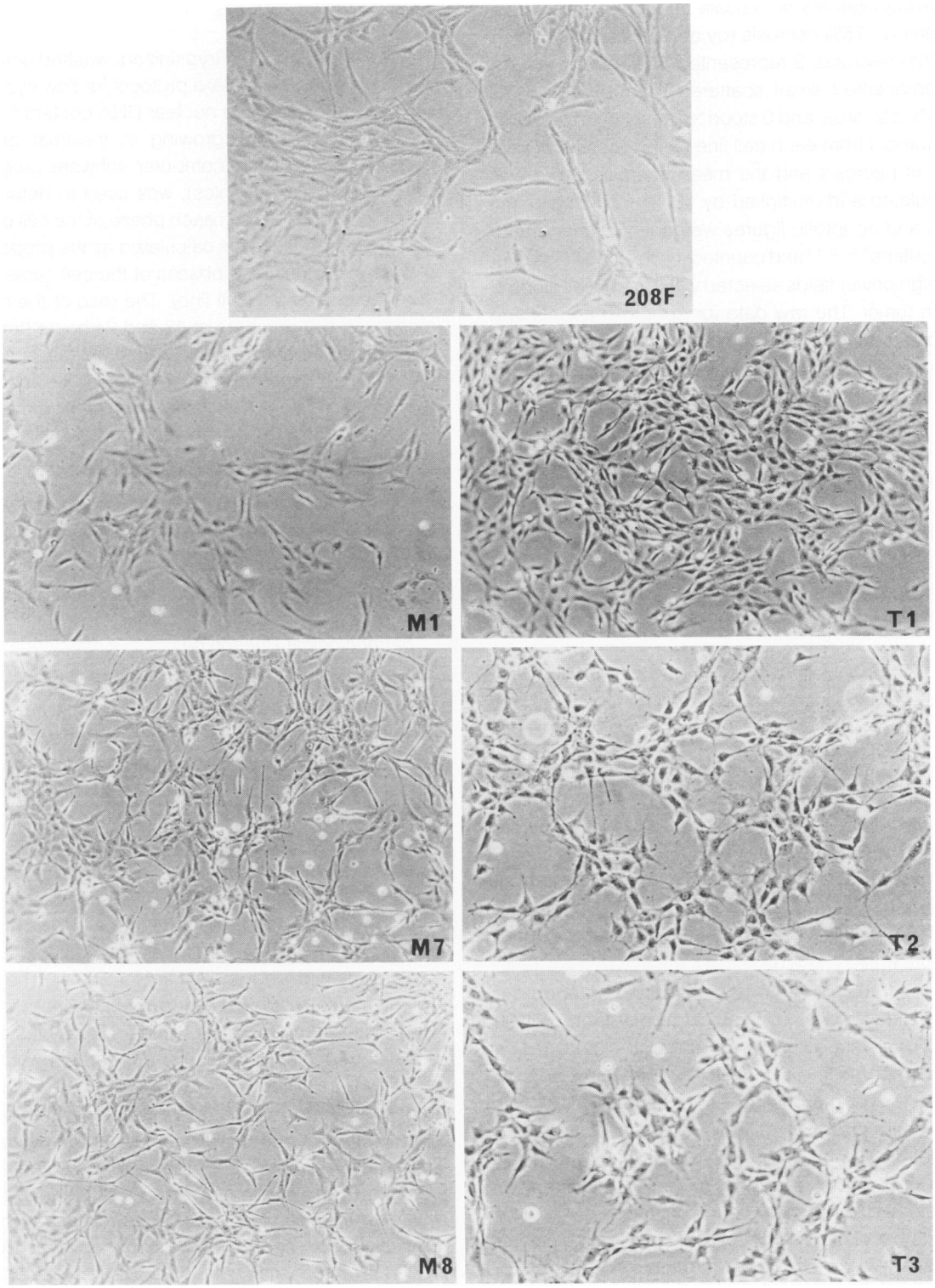

Figure 1. *Phase contrast photomicrographs of growth pattern in culture of the parental cell line 208F, the three* c-myc *transfectants M1, M7, and M8, and the three T24-*ras *transfectants T1, T2, and T3* (×250).

than untransfected 208F cells[39], and immunocytochemical analysis of p21^{ras} protein expression showed 10-fold higher levels for T1, 4-fold higher for T2 cells, and 2-fold higher for T3 cells compared with 208F controls.[35] The *myc*/*ras* cotransfectants MT9 and MT10 and the HPV transfectants H16 and H18 all

showed intermediate degrees of transformation with limited piling up of moderately refractile cells (data not shown).

Growth Fractions and Population Expansion

All transfectants had closely similar growth fractions in culture (Table 1). The only statistically significant differences were between M7 and MT9 ($P = 0.02$) for the oncogene transfectants and between H18 and H16R ($P < 0.05$) for the HPV-containing cell lines. However, PE values differed greatly between the 14 lines and showed a strong inverse correlation ($r = -0.65$) with the log AI in culture (regression equation log AI = 1.27 −0.349 PE; $P = 0.012$) (Figure 2).

Apoptosis

Transfected fibroblasts die in culture by apoptosis, as previously characterized ultrastructurally and by chromatin cleavage.[14,35] AI in culture varied over 60-fold between lowest and highest mean values. The three pure c-*myc* transfectants (M1, M7, and M8) showed significantly higher levels than the control 208F ($P < 0.05$ in all cases) (Table 1). The T24-*ras*-containing transfectant T1 and its derivative MT7 showed significantly reduced apoptosis compared with the control 208F ($P < 0.015$). T1, T2, and MT7 all had significantly less apoptosis than the three pure c-*myc* transfectants ($P < 0.025$). Of the lines derived from simultaneous co-transfection with *myc* and *ras*, MT10 had a similar log AI and MT9 a significantly higher log AI than 208F ($P < 0.01$), but both had significantly lower log AIs than the two pure *myc* transfectants M7 and M8 ($P < 0.05$). Among the HPV-containing lines, the only significant difference was between H18R and H18 ($P = 0.028$) due to reduced apoptosis associated with *ras* in combination with HPV 18 compared with HPV 18 alone.

Growth In Vivo

Malignant Phenotype

Earlier experiments[39] (unpublished observations) had established that the parental 208F line produces small indolent nodules, nonprogressive over periods of a month or more, whereas the oncogene transfectants usually generate tumors that grow at different rates but are eventually lethal. In the 12-day study period of the present experiments the T24-*ras*, c-*myc*, and HPV transfectants each formed invasive tumors in all of the six to seven injected mice. As expected the parent cells (208F) formed small nodules with no histological evidence of invasion of skin or muscle in 10 of 11 mice and no residual lesion in the 11th.

A/M Ratio

When apoptosis was studied relative to mitosis (A/M ratio) in the cell lines growing as tumors *in vivo* a pattern emerged similar to the observations of apoptosis *in vitro* (Table 1). The three pure c-*myc* transfectants (M1, M7, and M8) and 208F each had A/M tios that were significantly higher than those of the T24-*ras*-containing transfectants T1, T2, and MT7 ($P < 0.01$ for all comparisons). The simultaneous co-transfectants MT9 and MT10 had intermediate A/M ratios that were also significantly lower than 208F, M1, M7, and M8 ($P < 0.01$). Tumors formed by H16 had

Table 1. *Growth Parameters In Vitro and In Vivo*

Cell Line	Transfected Enhancer/ Oncogene	Growth Fraction *In Vitro*	Population Expansion *In Vitro*	Apoptosis Index (log) *In Vitro*	Tumor Size	Tumor Mitosis	Tumor Apoptosis	Tumor A/M Ratio (log)	Tumor Necrosis
208F	Parent	35.6	1.53	2.62 (0.42)	0.29	1.03	2.8	3.36 (0.53)	0
M1	Mo/***myc***	40.4	2.89	4.57 (0.66)	0.37	3.4	7.3	2.92 (0.47)	1.7
M7	Mo/***myc***	29.8	1.54	41.52 (1.62)	0.51	9.6	22.6	3.18 (0.50)	13.3
M8	Mo/***myc***	39.9	1.20	15.44 (1.19)	0.13	1.95	12.9	7.47 (0.87)	5.0
T1	SV/***ras***	40.1	3.56	0.77 (−0.11)	2.20	18.2	3.9	0.22 (−0.66)	31.7
T2	SV/***ras***	38.9	3.67	1.83 (0.26)	2.25	18.2	11.6	0.64 (−0.20)	21.7
T3	SV/***ras***	36.6	1.77	8.44 (0.93)	2.20	6.35	11.1	1.89 (0.28)	14.2
MT7	SV/***ras*** **and** Mo/***myc***	33.1	4.06	0.62 (−0.21)	2.42	26.1	4.1	0.17 (−0.77)	40.0
MT9	Mo/***myc*** **and** SV/***ras***	43.7	1.67	5.95 (0.78)	1.02	12.2	3.6	0.30 (−0.52)	12.5
MT10	Mo/***myc*** **and** SV/***ras***	40.8	2.30	2.30 (0.36)	1.42	16.1	10.9	0.69 (−0.16)	16.7
H16	Mo/HPV16	34.7	2.19	1.28 (0.11)	0.41	12.8	20.3	1.77 (0.25)	26.7
H16R	Mo/HPV16 and SV/***ras***	31.9	1.52	1.22 (0.09)	0.99	14.9	6.2	0.48 (−0.32)	28.3
H18	Mo/HPV18	42.9	1.71	1.56 (0.19)	1.05	16.7	11.5	0.69 (−0.16)	17.5
H18R	Mo/HPV18 and SV/***ras***	34.7	3.71	0.74 (−0.13)	0.92	17.0	3.1	0.19 (−0.73)	21.7

Transfected enhancer/oncogene sequences: Mo, Moloney virus long terminal repeat enhancer; SV, SV40 enhancer; *myc*, c-*myc* proto-oncogene; *ras*, mutated c-Ha-*ras*; oncogene; growth fraction, S+G2/M phases (ave. 9 to 10 expts.); population expansion (ave. 3 to 9 expts.); apoptotic index (ave. 5 to 10 expts.); tumor size (ave. 6 to 10 expts.); tumor mitotic counts (ave. 60 high power fields); tumor apoptotic counts (ave. 60 high power fields); A/M ratio was calculated by dividing counts/10 high power fields for each of 10 high power fields in 6 tumors (ave. 60); tumor necrosis extent assessed semiquantitatively using an arbitrary scale (ave. 6 tumors).

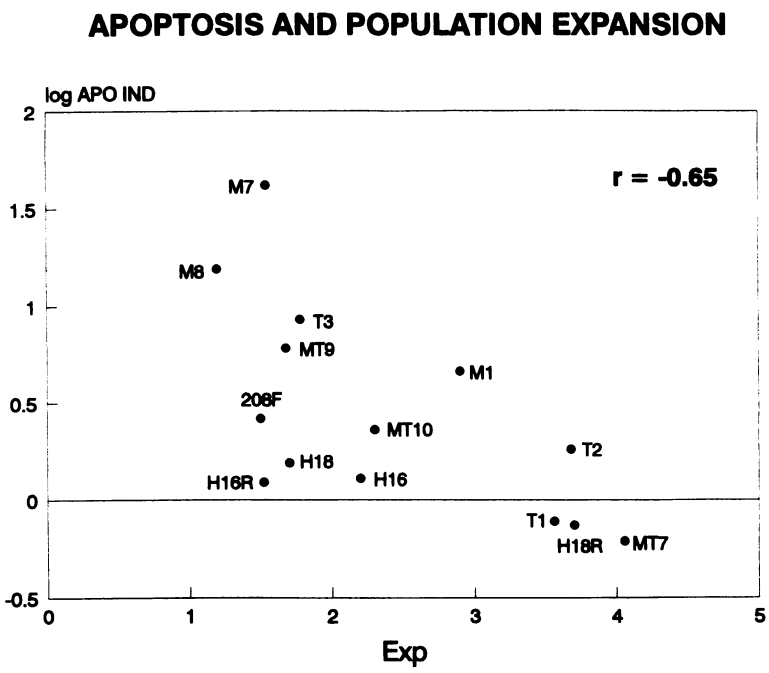

Figure 2. *Scatter plot of log AI* versus *population expansion for 14 oncogene and HPV-containing cell lines growing in culture. There is an inverse correlation (r = −0.65).*

significantly higher A/M ratios than those generated by H18 ($P < 0.005$). A/M ratios in H18R tumors were as small as those of T1 and MT7 and were significantly lower than those in H16 tumors ($P < 0.002$) and H18 tumors ($P < 0.004$). Unlike the results *in vitro*, however, the different transfectants showed divergent absolute mitotic counts. All exceeded that of the parental line and the highest three were all T24-*ras*-containing transfectants (T1, T2, and MT7), whereas the pure c-*myc* transfectants showed generally low values.

Apoptosis in culture, as measured by the log apoptotic index, correlated well (r = 0.73) with apoptosis relative to mitosis (log A/M) in tumors *in vivo* (regression equation log AI = 0.473 + 0.738 log A/M; $P = 0.003$). The three c-*myc* transfectants M1, M7, and M8 had high levels of apoptosis both *in vitro* and *in vivo*, whereas the T24-*ras*-containing lines T1, T2, and MT7 showed low values for both, and combinations of HPV/*ras*/*myc* were intermediate (Figure 3, A).

Tumor Size

All transfectants formed tumors that were larger in size than the indolent nodules formed by the parent cell line 208F. The sizes of the primary tumors formed by the T24-*ras* transfectants T1, T2, T3, and MT7 were significantly greater than those of the pure c-*myc* lines M1, M7, and M8 ($P < 0.01$), as were those of the two co-transfectants MT9 and MT10 ($P < 0.015$) (Table 1). Tumor sizes for the 14 lines showed an inverse correlation (r = −0.64) with log A/M ratios (regression equation tumor size = 1.11 − 0.973 log A/M; $P = 0.015$) (Figure 3B) and a positive correlation with absolute mitotic counts (r = 0.68; regression equation tumor size = 0.208 + 0.076 tumor mitosis; $P = 0.008$). Absolute mitotic count data should be interpreted with caution because potentially confounding topographical features (such as cell size and density) are not taken into account. Furthermore, population expansion in culture correlated positively (r = 0.61) with tumor size (regression equation PE = 1.52 + 0.741 tumor size; $P = 0.022$). Hence, the *ras*-containing transfectants T1, T2, and MT7 showed high levels of net growth both *in vitro* and *in vivo*, whereas the pure *myc* transfectants M1, M7, and M8 had low values of both, and the HPV/*ras*/*myc* combinations were mostly intermediate.

Tumor Necrosis

For all 14 cell lines, tumor necrosis score demonstrated a good correlation with absolute mitotic counts (r = 0.90; regression equation necrosis = 0.18 + 1.42 tumor mitosis; $P < 0.0001$) (Figure 4A) and with tumor size (r = 0.65; regression equation tumor size = 0.333 + 0.0459 necrosis; $P = 0.012$) but a strong inverse correlation with tumor apoptosis relative to mitosis (r = −0.75; regression equation necrosis = 17.2 − 16.3 log A/M; $P = 0.002$) (Figure 4B). Furthermore, tumor necrosis score correlated inversely with measures of apoptosis in culture (r =

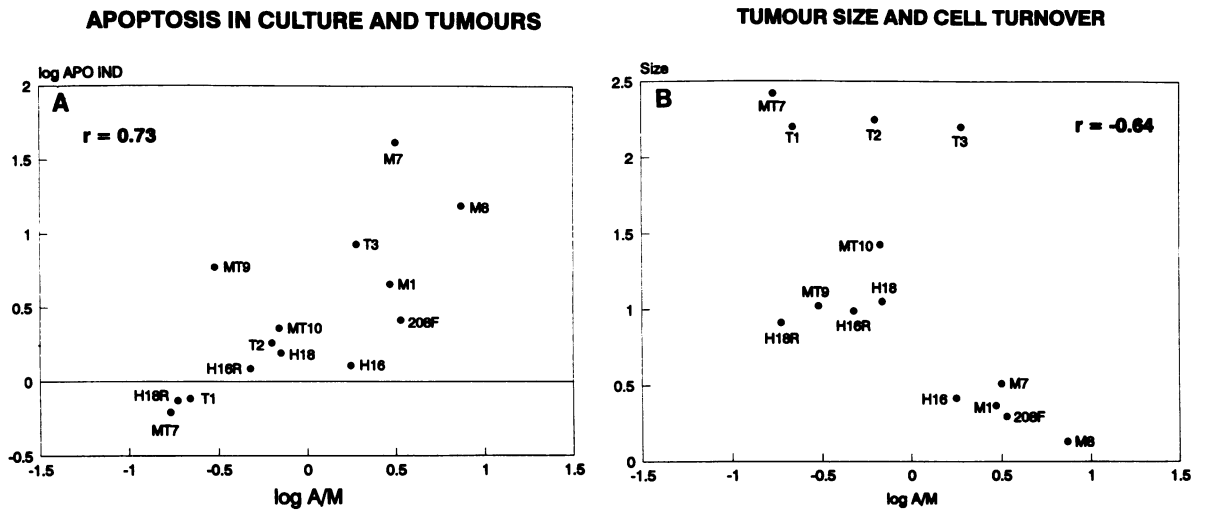

Figure 3. *Scatter plots of apoptosis relative to mitosis (log A/M ratio) in tumors* versus (A) *apoptosis in culture expressed as log AI (correlation r = 0.73) and* (B) *tumor size (correlation r = −0.64).*

−0.66; regression equation necrosis = 24.2 − 14.2 log AI; $P = 0.01$) (Figure 4C). Thus, the extent of necrosis appears to reflect both tumor size and proliferation rate *in vivo* but nevertheless is strongly inversely related to apoptosis.

Relationship Between Apoptosis and Necrosis in the Tumor Microenvironment

The data presented above demonstrate the negative correlation between extent of necrosis and prevalence of apoptosis in the tumors formed by the different cell lines. In these evaluations care was taken to measure apoptosis only in viable parts of the tumors (Figure 5, A and B) distant from necrotic regions. In the course of this assessment, however, we noticed that the zones of confluent necrosis observed in the tumors were often surrounded by a rim of viable tumor cells densely admixed with apoptotic cells. The size of the necrotic zone and the thickness of the apoptotic rim tended to vary depending on the transfected gene. The group of T24-*ras* lines T1, T2, T3, and MT7 formed large tumors containing extensive areas of confluent necrosis with only a thin rim of apoptosis at the interface between viable and necrotic tissue. Overall, their necrosis scores were moderate or high (Table 1). In contrast, in the 12-day time interval studied, the three pure c-*myc* transfectants M1, M7, and M8 generated small tumors that showed only occasional small zones of necrosis and these were surrounded by thick rims of apoptosis at the viable necrotic boundaries. The HPV/*ras*/*myc* combinations were mostly intermediate in tumor size and propensity for necrosis with apoptotic rims of variable thickness.

To study this further, M1 and T1 tumors were compared in animals that had been left undisturbed until comparable tumor sizes were achieved. T1 tumors were harvested between 8 and 12 days after inoculation, whereas M1 tumors of comparable size were only achieved after various periods up to 70 days. In this experiment, 21 mice were injected with M1 cells and left for 12 to 70 days. Of these, 13 mice developed tumor nodules of which 6 were small (less than 1 cm in maximum dimension) and showed no significant necrosis but 7 were of large size, similar to T1 8- to 12-day tumors. Five of the seven large M1 tumors did contain zones of necrosis and in every case the edge of the necrotic zone showed large numbers of apoptotic cells (Figure 5C). The five large M1 tumors containing necrotic zones were analyzed for tumor apoptosis and mitosis in their viable zones; apoptotic counts increased to higher levels than those of the standard 12-day M1 tumors (mean tumor apoptosis 20.0) as did tumor mitosis (mean 13.0) but the A/M ratio decreased slightly to 1.66. In contrast, apoptotic cells were rare in T1 tumors even at the interface between viable and necrotic regions (Figure 5D). Thus, the extent of apoptosis at the viable necrotic boundaries appeared to reflect the susceptibility to apoptosis in the viable regions elsewhere in the tumor.

Discussion

Apoptosis Has a Major Influence on Net Tumor Growth Rate

The data confirm the importance of apoptosis in determining the growth of tumor cell populations. Analy-

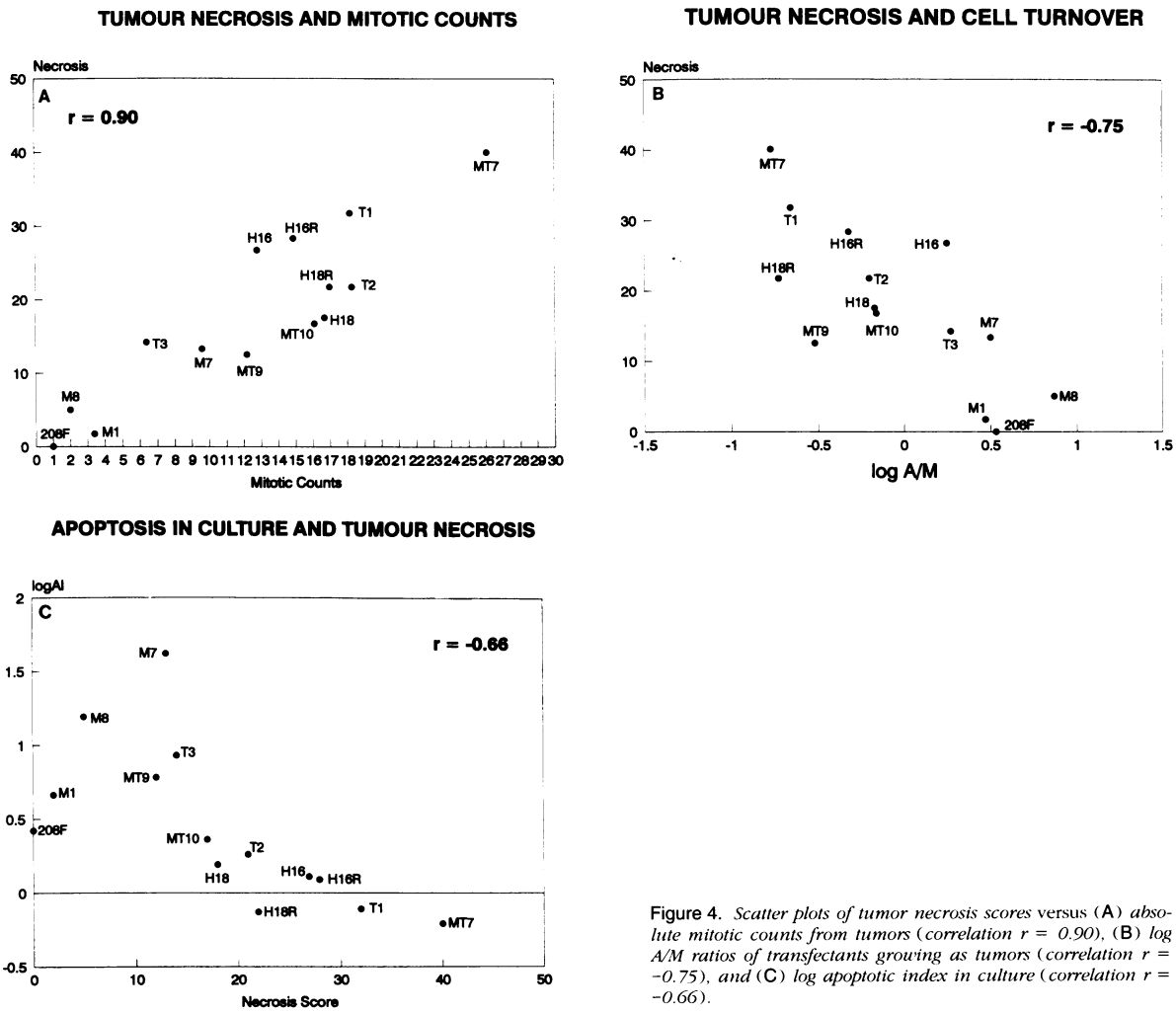

Figure 4. *Scatter plots of tumor necrosis scores* versus (A) *absolute mitotic counts from tumors (correlation r = 0.90),* (B) *log A/M ratios of transfectants growing as tumors (correlation r = −0.75), and* (C) *log apoptotic index in culture (correlation r = −0.66).*

sis of growth parameters of the 14 cell lines in culture revealed similar growth fractions in the different transfectants but widely divergent rates of cell population expansion and apoptosis. Population expansion and apoptosis showed a statistically significant inverse relationship. Thus, the rate of apoptosis appeared to be the major variable determining differences in the rate of population expansion *in vitro*. The sizes of tumors formed *in vivo* by these transfectants also correlated with their population expansion *in vitro* and showed an inverse correlation with A/M ratios *in vivo*. Histologically, all were moderate or poorly differentiated fibroblastic tumors, thus differentiation, as a means of exit from the proliferating pool, was most unlikely to be significant. The ratio of apoptosis to mitosis reflects changes in both numerator and denominator and should be interpreted with caution but it has been used successfully by others to study frequency of apoptosis in histological sections.[42] The fact that the rates of apoptosis *in vitro* correlated closely with the A/M ratio in tumors *in vivo* suggested to us that the rate of cell turnover within these tumors was determined by intrinsic susceptibility to apoptosis rather than by environmental factors alone.

Susceptibility to Apoptosis may Determine the Probability of Tumor Necrosis

The extent of necrosis in tumors is difficult to measure accurately and objectively and was assessed only semiquantitatively in this study. Necrosis showed a positive correlation with tumor size and prevalence of mitosis in tumors but an inverse correlation with the ratio of apoptosis to mitosis *in vivo* and with its *in vitro* homologue, AI. There appeared to be a spectrum of the pattern of cell death within this family of tumors. In some tumor types death occurred mostly by apoptosis with little necrosis. This was seen in slowly

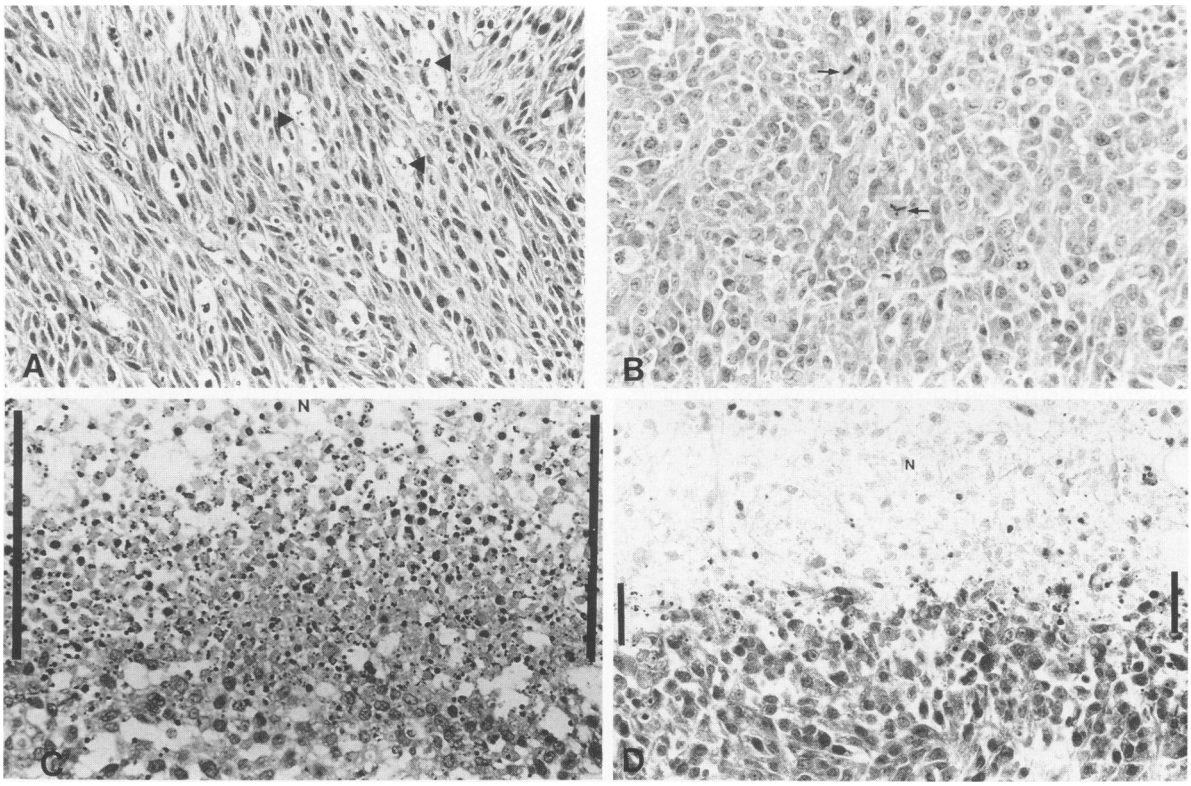

Figure 5. *Photomicrographs of the patterns of tumor death from* (**A**) *a c-*myc *transfectant* (*M7*) *viable tumor region displaying frequent apoptosis* (**arrow heads**) *relative to mitosis,* (**B**) *a T24-*ras *transfectant* (*T1*) *viable tumor region showing plentiful mitotic* (**arrows**) *but few apoptotic figures,* (**C**) *a c-*myc *transfectant* (*M1*) *large tumor showing a small area of necrosis* (*N*) *with considerable apoptosis* (**zone between bars**) *at the boundary region; and* (**D**) *a T24-*ras *transfectant* (*T1*) *displaying a large zone of confluent necrosis* (*N*) *with only a thin rim of apoptosis* (**zone between bars**) *at the necrotic viable interface* (*hematoxylin and eosin, ×250*).

growing tumors, as exemplified by those formed by *myc* transfectants. At the other end of the spectrum, tumor cells died largely by necrosis with little apoptosis, a pattern observed in rapidly growing tumors such as those formed by *ras* transfectants. HPV/*ras*/*myc* combinations displayed intermediate properties.

A simple explanation for this inverse relationship between apoptosis and necrosis in tumors is that the intrinsic cellular susceptibility to apoptosis determines the probability of necrosis. Cellular sensitivity or resistance to apoptosis in response to one type of injury stimulus is often associated with similar sensitivity or resistance to a variety of other agents.[28,43–50] We have argued elsewhere that this is because some cell types are "primed" for apoptosis.[14] Indeed, we have already shown in some of the cell lines studied here that those most and least susceptible to apoptosis differ in their content of a nuclear endonuclease activity that may be one of the effector proteins of apoptosis.[35]

We therefore propose that during growth *in vivo* tumor cells that are primed for apoptosis are liable to die by apoptosis in adverse conditions such as mild ischaemia, reduction in availability of essential substrates and growth factors, or falling pH. In contrast, cells that are intrinsically less susceptible to apoptosis might be more resistant to these conditions and therefore survive, at least initially, within deviant tumor microenvironments. Low susceptibility to apoptosis is thus likely to associate with both rapid expansion of the tumor cell population and the development within the tumor of zones of hypoxic but viable tumor cells. Eventually, necrosis is the obligate mode of death of these cells, whether provoked by the progressive imbalance between growth of the tumor and that of its supporting blood supply, or by more acute vascular events (compression, thrombosis, or spasm). We made no attempt to measure angiogenesis or blood flow directly but this proposition is supported by the exceptionally high incidence of apoptosis at the viable/necrotic boundary in those c-*myc*-transfected tumors in which necrosis did occur, in contrast to its low incidence at similar locations in *ras*-expressing tumors of similar size.

One alternative explanation for the inverse relationship between apoptosis and necrosis might be that

necrosis in these subcutaneous rodent fibroblast tumors is simply determined by tumor size. Two independent observations argue against this. First, the extent of necrosis does not automatically reflect tumor size. Tumors of very similar sizes such as MT7, T1, T2, and T3 had mean necrosis scores varying from 14.2 to 40. Second, the c-*myc* tumors in the series that were allowed to grow to large size still showed less necrosis than *ras* tumors of comparable size. Thus, although the ratio of tumor volume to functional vascular supply is undoubtedly important in determining which tumor zones become hypoxic, the hypothesis advanced here does not rest exclusively on the time-honored notion that proliferating tumor cells outgrow their blood supply. Rather, it indicates that cellular susceptibility to apoptosis may prevent development of a severely deviant microenvironment and therefore limit the extent of necrosis.

Oncogenes Determine Patterns of Tumor Death

The preceding paragraphs have emphasized the intrinsic differences in apoptotic rates in the various transfected derivatives of 208F and the significance of this in determining the overall tumor growth rate and, perhaps, the conditions of the tumor microenvironment that favor or discourage necrosis. The question arises whether these differences in apoptosis are themselves determined by the expression of the transfected oncogenes. Although the presence of the transfected genes was confirmed in all these cell lines (and their expression in many), in experiments of this design it is seldom possible to completely exclude the possibility that critical but unidentified cellular changes have been co-selected with expression of the transfected gene during clonal expansion of the cell line *in vitro* and *in vivo*. However, striking similarities emerged between independent transfectants expressing the same oncogene. In particular, pure c-*myc* transfectants tended to be of the slow-growing, high A/M ratio, low necrosis type, whereas the pure *ras* transfectants showed the faster-growing, low A/M ratio, extensive necrosis phenotype.

The intermediate position of most of the *ras*/*myc*/HPV co-transfectants may suggest gene dose-dependent interactions. Recent data indicate that constitutive c-*myc* expression in fibroblasts initiates apoptosis under conditions of suboptimal growth support, such as reduced serum growth factor exposure *in vitro*.[35,39,51–53] Under these circumstances, expression of genes such as *bcl-2*, *abl*, and certain viral oncogenes can rescue the *myc*-expressing cells at the same time rendering them resistant to a variety of pharmacologically unrelated lethal stimuli *in vitro*.[46,48–50,54–57] The data presented here strongly suggest that constitutive c-*myc* expression has entirely analogous effects in fibroblastic tumors growing *in vivo*, and that expression of mutated Ha-*ras*, especially at the high levels that are found in the T1 subline,[33,35,39] has a survival effect similar to that of *bcl-2*.

The two high risk HPV types were associated with moderate to high levels of tumor cell apoptosis compared with HPV/*ras* co-transfectant tumors, and it is interesting that expression of the HPV-transforming genes may stimulate *myc* transcription. Binding of the retinoblastoma protein p105Rb (Rb) by HPV 16/18 E7[58–60] has been reported to release Rb-mediated repression of c-*myc*.[61–63] Rb itself can inhibit apoptosis in certain circumstances.[64,65] However, binding of HPV 16/18 E6 to p53, directing its rapid degradation,[66] may block stimulation of apoptosis by wild-type p53.[30,67] Thus, HPV 16 and 18 genomes, which are strongly implicated in the development of cervical cancer,[37,38,68] may set in train many pathways, including *myc* activation and p53 degradation, that might have opposing effects on apoptosis.

It is clear that not all the phenotypic effects of oncogene transfection are explicable in terms of changes in apoptosis alone. In *ras* transfectants, for example, there is a consistent increase in the frequency of mitotic events *in vivo* that is not evident *in vitro*. Presumably, growth *in vitro* is rendered independent of some of the cell proliferative effects of the transfected *ras* gene through exogenous supply of growth factors in the medium. Nonetheless, the data presented here support the view that oncogene expression materially influences both the intrinsic susceptibility to apoptosis of tumor cells and its relationship to cell proliferation and hence affects fundamental features of the mode and rate of growth *in vivo* and the character of the tumor microenvironment.

The inverse relationship between apoptosis and necrosis, in particular the association of low susceptibility to apoptosis with fast net growth and extensive necrosis, affords a rational basis for empirically derived tumor grading systems that place emphasis on the extent of necrosis as an indicator of poor prognosis in many human tumors including soft tissue sarcomas.[3,4,22] Inclusion in such grading systems of an indicator of tumor cell susceptibility to apoptosis, such as absolute apoptotic levels, or A/M ratios, or detection of preformed effector molecules of apoptosis such as endonuclease activity,[69,70] or *in situ* end labeling of fragmented DNA,[15] may hold promise.

Furthermore, because susceptibility or resistance of tumor cells to induction of apoptosis by chemotherapeutic drugs or irradiation appears also to be genetically determined,[27,29,30,31,46,71] such a modified grading system that gives an indication of susceptibility to apoptosis may also be useful in predicting response to therapy.

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